JOURNAL OF BACTERIOLOGY, Apr. 1974, p. 231-241

Vol. 118, No. 1
Printed in U.S.A.

# Asparagine Utilization in *Escherichia coli*

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Received for publication 29 October 1973

Asparagine-requiring auxotrophs of *Escherichia coli* K-12 that have an active cytoplasmic asparaginase do not conserve asparagine supplements for use in protein synthesis. Asparagine molecules entering the cell in excess of the pool required for use of this amino acid in protein synthesis are rapidly degraded rather than accumulated. Supplements are conserved when asparagine degradation is inhibited by the asparagine analogue 5-diazo-4-oxo-L-norvaline (DONV) or mutation to cytoplasmic asparaginase deficiency. A strain deficient in cytoplasmic asparaginase required approximately 260 μmol of asparagine for the synthesis of 1 g of cellular protein. The cytoplasmic asparaginase (asparaginase I) is required for growth of cells when asparagine is the nitrogen source. This enzyme has an apparent $K_m$ for L-asparagine of 3.5 mM, and asparaginase activity is competitively inhibited by DONV with an apparent $K_i$ of 2 mM. The analogue provides a time-dependent, irreversible inhibition of cytoplasmic asparaginase activity in the absence of asparagine.

The existence of asparagine-requiring auxotrophs of *Escherichia coli* (5) establishes that asparagine can enter this organism and be maintained without degradation. However, the relatively high concentrations of asparagine reported to be required for supplementation of asparagine-requiring auxotrophs (4) and for repression of the synthesis of the asparagine synthetase in the wild-type organisms (5) has led to the suggestion that this amino acid may not be accumulated by *E. coli* (4, 5). This implies that the mechanism responsible for asparagine entry is not saturated at concentrations comparable with those that saturate the systems responsible for the uptake of the other amino acids. However, these observations could also be explained by the extensive catabolism of the amino acid after entry, as has been observed with aspartate (11), glutamate (6), and glutamine (21).

This study examines the utilization of asparagine supplements of cultures growing in the presence of 5-diazo-4-oxo-L-norvaline (DONV), an asparagine analogue which is a potent, irreversible inhibitor of the periplasmic asparaginase (9), and utilization by a mutant selected from a population of the asparagine auxotroph that was unable to utilize asparagine as a nitrogen source.

A portion of this paper was submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree in Biological Sciences from the University of California, Irvine.

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## MATERIALS AND METHODS

**Chemicals.** Uniformly labeled L-[$^{14}$C]asparagine (218 μCi/μmol) was obtained from Amersham/Searle, Des Plaines, Ill.

The asparagine analogue, DONV, was a gift from R. E. Handschumacher, Department of Pharmacology, Yale University, New Haven, Conn. L-β-Hydroxamyl aspartic acid and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, Mo. All other materials used in this study were obtained from the usual commercial sources and were either reagent grade or the highest chemical purity available.

**Bacterial strains.** The strains used were derivatives of *E. coli* K-12. Strain 25287, an asparagine, thiamine auxotroph originally isolated by E. Reich of Rockefeller University, New York, was obtained from the American Type Culture Collection. Cedar and Schwartz (5) have demonstrated by enzymatic and genetic characterization that strain 25287 lacks asparagine synthetase activity. A mutant, 47, was isolated from strain 25287 after ultraviolet mutagenesis and penicillin selection (13) for the inability to use L-asparagine as nitrogen source. Cultures were cloned on nutrient broth agar plates monthly and screened to avoid revertants by a check of specific growth requirements on solid media.

**Media.** Cultures were grown in the synthetic minimal medium 56 described by Monod et al. (15), supplemented with 0.4% glucose, 5 μg of thiamine per ml and, unless stated otherwise, 150 μg of L-asparag-

ine per ml. Where noted, L-asparagine or L-aspartate replaced ammonium sulfate as the nitrogen source. The nutrient broth medium has been described by Salivar et al. (19) but contained in addition 2 mM L-asparagine, 2 mM L-glutamine, and 1% glycerol instead of glucose. The asparagine and glutamine used in media were sterilized by filtration through 0.22-$\mu$m membrane filters (Millipore Filter Corp., Bedford, Mass.). All other culture materials were sterilized by autoclaving. Solid media were prepared with 1.0% agar-agar no. 3 (Oxoid-Colab, Chicago Heights, Ill.).

**Growth of cells.** Liquid cultures were initiated from single-colony isolates transferred into tubes of broth. Portions of cells from broth cultures were twice grown to saturation in minimal media before use in the experiments described. Cultures were grown at 37 C in a Gyrorotary shaking water bath (New Brunswick Scientific Co., Inc., New Brunswick, N.J.) oscillating 250 times per min. Triple-baffled, nephlo-type growth flasks (Bellco Glass, Inc., Vineland, N.J.) were used, and the volume of the culture never exceeded 20% flask volume. Subcultures were, unless noted otherwise, started with 2% inoculum of cells washed twice with wash medium (minimal medium minus carbon and nitrogen sources) by centrifugation at room temperature. This wash procedure was usually completed in 15 min. Growth was followed with a Klett-Summerson photometer equipped with a no. 54 filter. A reading of 100 Klett units corresponds to 0.5 mg of cells (dry weight) and 0.25 mg of protein per ml. Growth rates were determined graphically from the slopes of semilogarithmic plots of Klett units versus incubation time.

Anaerobic growth conditions were approached by autoclaving a screw-cap Erlenmeyer flask filled to capacity with medium. The flasks were lightly sealed and allowed to equilibrate to 37 C undisturbed. A 1% inoculum was used in the experiments described and, after inoculation, the flasks were again lightly sealed and left undisturbed at 37 C until harvest.

**Mutagenesis and selection of asparaginase-deficient mutants.** Approximately 2 ml of an early exponential phase culture of strain 25287 growing in minimal medium was layered over the bottom of a petri dish. The cells were then irradiated for 90 to 120 s with an UVS-11 ultraviolet lamp (Ultraviolet Products, San Gabriel, Calif.) placed at a distance of 10 cm above the bottom of the open petri dish. Through the use of a killing curve prepared for *E. coli* K-12 3110 with this ultraviolet source, it was estimated that the mutagenesis procedure resulted in 0.1 to 0.01% survival. Portions of the irradiated culture were transferred to nutrient broth and allowed to grow to saturation at 37 C (22).

The cells were washed twice by centrifugation at room temperature with wash medium and suspended to a concentration of $2 \times 10^8$ cells/ml in minimal medium without nitrogen sources. Residual growth was followed until the cells were nitrogen starved. The starved cells were diluted with 60% sucrose to yield a 20% sucrose suspension, and L-asparagine and penicillin (prepared in the minimal medium without nitrogen source but containing 20% sucrose) were then added to final concentrations of 20 mM and 3,000 units per ml, respectively. Growth was followed by direct microscopic examination until greater than 50% spheroplast formation was observed. Portions were then diluted with minimal medium without nitrogen source, and samples corresponding to 100 to 300 viable cells were spread on nutrient agar plates.

The plates were incubated at 37 C until colony formation was observed and then replicated to minimal media plates containing 0.4% glucose and one of the following nitrogen sources at 10 mM final concentration: ammonium sulfate, L-aspartate, or L-asparagine. The replica plates were incubated at 37 C for 24 to 36 h. Colonies that possessed the phenotype of growth on plates containing ammonium sulfate and L-aspartate but not L-asparagine were located on the master plate and rescreened in a similar manner. Those colonies for which the phenotype persisted were tested further as described in Results.

**Assays.** Asparaginase I and II were assayed by methods similar to those described by Schwartz et al. (20). Total asparaginase activity was assayed by measurement of the ammonia formed at 37 C in 15 min. The assay mixture (1.0 ml) contained 10 mM L-asparagine, 0.1 M tris(hydroxymethyl)aminomethane (Tris-$KH_2PO_4$ [pH 8.0] and 1 $\mu$M $MgCl_2$. The reaction was terminated by addition of 50 $\mu$liters of 100% trichloroacetic acid. Ammonia was determined on 0.5-ml portions of the centrifuged sample supernatant by direct nesslerization. Asparaginase II activity was assayed by measurement of [$^{14}$C]aspartate formed at 37 C in 30, 60, and 90 s. The assay mixture (0.06 ml) contained 0.1 mM L-[$^{14}$C]asparagine (uniformly labeled, specific activity 20 $\mu$Ci/$\mu$mol), 0.1 M Tris-$KH_2PO_4$, and 1 $\mu$M $MgCl_2$. The reaction was terminated by dilution of 10 $\mu$liters of reaction mixture with 10 $\mu$liters of unlabeled 10 mM L-asparagine, and the entire mixture was spotted directly onto Whatman ET81 Ecteola cellulose ion exchange paper warmed from beneath with a hot-air blower. Aspartate was separated from asparagine by descending elution of the paper with 0.1 M acetic acid. The area bearing aspartate was isolated with the aid of parallel chromatographed standards and radioactivity measured by scintillation counting in 2 ml of Aquasol (New England Nuclear Corp., Boston, Mass.). Asparaginase I was estimated as the difference of total activity measured by ammonia formation at 10 mM and by asparatate formation at 0.1 mM (20).

**Analytical methods.** Protein concentration was determined by the method of Lowry et al. (12) using bovine serum albumin as the standard. Measurements of radioactivity were made in an LS-100 scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). Either Aquasol or a toluene-based solution containing 0.85% Fluoralloy TLA and 1% BBSIII solubilizing agent (Beckman Instruments, Inc.) were used as scintillation fluids.

Ammonia was measured by adding 0.5 ml of sample to 3.0 ml of 12.5% Nesslers reagent (45.5 g of $HgI_2$ and 35.0 g of KI in 1 liter containing 112 g of KOH) (14). After 10 min, the absorbance was mea-

sured with a Klett photometer equipped with a no. 42 filter.

**Preparation and identification of materials in protein hydrolysates.** Protein fractions of cells prepared by hot, trichloroacetic acid precipitation were washed twice by suspension and centrifugation in acid ethanol as described by Roberts et al. (18).

The distribution of radioactive label in the protein hydrolysates of cells grown in the presence of L-[$^{14}$C]asparagine was determined by a modification of the method described by Roberts et al. (18). A 4.0-ml sample of culture growing in the presence of labeled asparagine was mixed with 1.0 ml of 50% trichloroacetic acid and incubated at 100 C for 30 min. The precipitated material was collected by centrifugation at 16,000 × *g* for 15 min and washed twice by suspension and centrifugation in acid-ethanol solution. The washed pellet was suspended in 10 ml of 6 N HCl and hydrolyzed by autoclaving in a sealed Pyrex tube for 12 h. The hydrolysate was evaporated to dryness and extracted twice with ether. The dry, ether-extracted protein hydrolysate was suspended in 50% methanol, and the concentration of the ninhydrin-positive material was adjusted to 0.1 M after determination by the method of Moore and Stein (16).

Protein hydrolysates were fractionated by one- and two-dimensional thin-layer chromatography. The radioactive areas of the chromatograms were then located by autoradiography with 14 days of film exposure. The radioactive compounds were identified by comparison of relative migrations with similarly prepared unlabeled samples and samples selectively reinforced with labeled amino acids. Radioactivity was determined by scraping the area of interest from the chromatogram and scintillation counting of the scraped sample in 2 ml of toluene scintillator.

Thin-layer chromatography was performed with 250-μm surface depth silica gel G plates (Brinkmann Instruments, Inc., Westbury, N.Y.). Solvent system I contained chloroform-methanol-ammonium hydroxide-water (8:8:3:1). Solvent system II contained propanol-water (1:1).

## RESULTS

**Growth studies with L-asparagine as an amino acid supplement.** Strain 25287 can establish a doubling time of 60 to 70 min in minimal medium provided the initial concentration of the asparagine supplement exceeds 25 μM (Fig. 1A). The results shown were obtained when inoculum was cultured in media with initial asparagine supplements of 50 to 100 μM. When the inoculum was precultured in media containing a higher concentration of asparagine, 1.5 mM, growth was observed only after long lags in the subcultures receiving supplements of 200 μM asparagine or less (Fig. 1B).

The ability of strain 25287 to grow without a lag in media containing low concentrations of asparagine, once established, is lost with a single passage of the cells in media containing 1.5 mM L-asparagine. This ability appears to be related to derepression of a high-affinity ($K_m$ = 3 μM) L-asparagine transport system by growth in the absence of high extracellular asparagine concentrations (R. C. Willis and C. A. Woolfolk, manuscript in preparation).

**Growth studies with L-asparagine as a nitrogen source.** Cultures of *E. coli* K-12 can utilize either asparagine or the deamidation products, aspartic acid and ammonia, as nitrogen sources. These compounds can be interchanged as sole nitrogen sources without any observable lags or shifts in growth rates characteristic of the adaptation to low asparagine concentrations reported above (Fig. 1). Roberts et al. (18) have observed that ammonia or aspartate can spare the utilization of asparagine as a nitrogen source, which suggests that this amino acid may not be a preferred source of nitrogen for *E. coli*. Kay (11) and we (data not shown) have observed that 1 mM L-aspartic acid can maintain a 70-min generation time for cultures utilizing this amino acid as the nitrogen source. The generation time for cultures utilizing L-asparagine as the nitrogen source is,

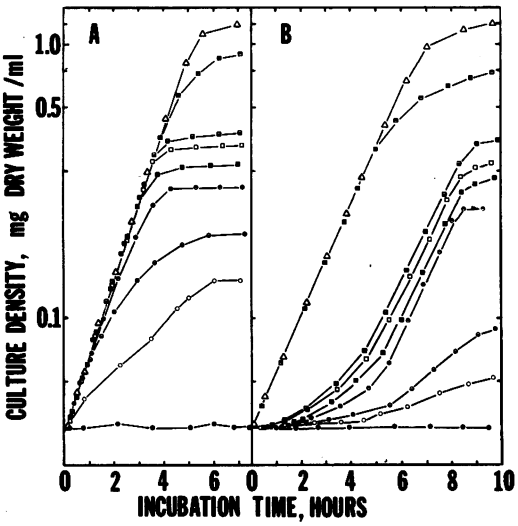


FIG. 1. *Growth behavior of an asparagine auxotroph. Strain 25287 was precultured under standard conditions in minimal medium initially supplemented with 0.05 mM L-asparagine (A) and 1.5 mM L-asparagine (B). The cells were washed and inoculated into minimal media supplemented with L-asparagine at initial concentrations of (△) 1.0 mM, (▣) 0.5 mM, (▣) 0.2 mM, (□) 0.1 mM, (■) 0.075 mM, (◓) 0.050 mM, (◔) 0.025 mM, (○) 0.010 mM, (●) no asparagine. Growth was continued under standard conditions and monitored at the times indicated.*

however, dependent on asparagine concentration below 5 mM, and the minimum generation time observed is 105 min (Fig. 2). The inability of cultures to use asparagine as rapidly as aspartate or ammonia as a nitrogen source could be due either to a slow rate of entry of the amino acid or an inadequate level of asparaginase activity.

A comparison of the generation times, expressed as the rate of nitrogen utilization by cultures (6), with the rate of asparaginase activity, measured at corresponding asparagine concentrations with toluenized cells (Table 1), suggests that asparaginase activity does not limit the growth rate of asparagine-nitrogen dependent cultures. Therefore, the growth rate of cells utilizing asparagine as the nitrogen source is apparently dependent on the rate of entry of the amino acid.

**Characterization of a cytoplasmic asparaginase-deficient mutant.** The selection procedure described in Materials and Methods incorporates several observations described above. Penicillin enrichment was initiated only after the generation time indicated that mutagenized cultures were dependent on asparagine for nitrogen. Culture of strain 25287 with 10 mM L-asparagine as the nitrogen source represses the high-affinity L-asparagine transport system (Willis and Woolfolk, manuscript in preparation). Entry of asparagine, which is required by this auxotroph, would then be mediated by only the system supplying the cells nitrogen requirements, and permeability mutants could be selected against during the screening procedure. Furthermore, cells were cultured with vigorous shaking in minimal media containing glucose as the carbon source to prevent formation of the catabolite-repressible periplasmic asparaginase activity (3, 20). Four mutants with the desired phenotype were isolated after screening 10,000 colonies. Of the four, only strain 47 was stable

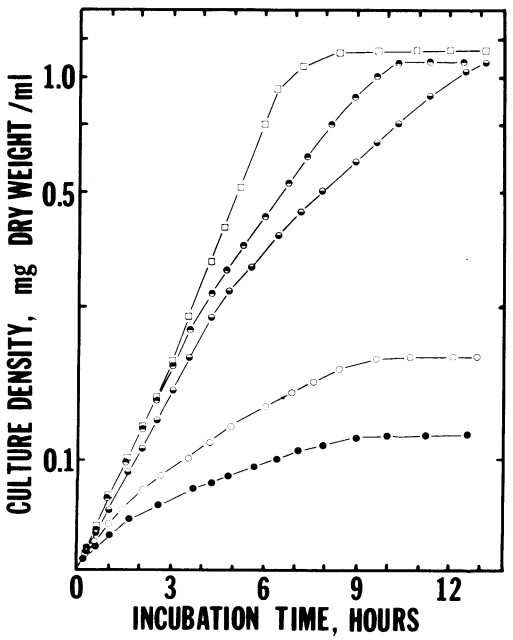


FIG. 2. *Asparagine as a nitrogen source. A culture of mid-exponential phase 25287 cells grown in minimal media was harvested, washed, and inoculated into minimal media containing* L-*asparagine as sole nitrogen source at concentrations of* (□) *25 mM,* (◓) *10 mM,* (◒) *5 mM,* (○) *2.5 mM, and* (●) *1 mM. Growth was continued under standard conditions.*

TABLE 1. *Comparison of the asparaginase activity required for asparagine-nitrogen dependent growth rates of cultures to the asparaginase activity of toluenized cells*

| Initial asparagine concn (mM) | Total asparaginase activity (μmol per h per mg) | |
|---|---|---|
| | Predicted[a] | Measured[b] |
| 25.0 | 3.25 (1.75) | ND |
| 20.0 | ND | 7.0 |
| 10.0 | 3.25 (1.75) | 7.0 |
| 5.0 | 2.97 (1.87) | 5.5 |
| 2.5 | 1.93 (2.85) | ND |
| 2.0 | ND | 3.5 |
| 1.0 | 1.23 (4.50) | 2.0 |
| 0.5 | ND | 1.3 |

[a] The predicted minimal rate of asparaginase activity required to support the doubling time observed at the indicated initial asparagine concentrations was calculated according to the following relationship: 0.693 (1/Dt[initial doubling time of culture]) (nitrogen mass factor); the nitrogen mass factor was 8 μmol of nitrogen/mg of cells (dry weight) (6, 18) and Dt, shown in parentheses, was determined from growth curves described in Fig. 2.

[b] The asparaginase assays were performed with washed, toluenized (10) cells as described in Materials and Methods. Ammonia formation was measured at initial concentrations of asparagine from 25 to 1 mM, and aspartate formation was measured at initial concentrations of asparagine from 2.0 to 0.5 mM. The level of background Nessler-positive material interfered with the detection of net ammonia formation in assays performed with initial concentrations of asparagine below 2 mM. The radioactive assay was used to extend the asparaginase assay to the lower concentrations. The average specific activities from three determinations of asparaginase activity by ammonia formation and aspartate formation at the 2 and 1 mM asparagine concentrations differed by 9 and 16%, respectively. Specific activity values shown are the average of at least three determinations.

enough for further study.

Table 2 summarizes results of asparaginase assays with strain 47 and the parent strain 25287. Activity attributable to the cytoplasmic asparaginase, asparaginase I, was not detected in strain 47 cultured under standard conditions and assayed by ammonia formation with 10 mM asparagine. Strain 47 does, however, retain the capacity to express the asparaginase II activity when grown anaerobically in nutrient broth. Interestingly, the total asparaginase activity of strain 47 cultured in the absence of conditions favoring catabolite repression is equivalent to that of the parent strain.

**Utilization of asparagine supplements for growth.** The growth response of the parent strain 25287 and the asparaginase-deficient mutant 47 to supplementation of their auxotrophy with asparagine is shown in Fig. 3. The cell mass achieved by cultures of strain 47 with variation of the initial concentration of asparagine supplement over a range of 0.010 to 100 mM approximates a linear function. The slope of the line describing the variation in strain 47 culture yield with asparagine concentration shown in Fig. 3 indicates that this strain requires 131 nmol of asparagine per mg of cells (dry weight). In contrast, cultures of strain 25287 achieved lower cell yields when provided with similar concentrations of asparagine supplements, suggesting that strains with an active cytoplasmic asparaginase use their asparagine supplements less efficiently. As described above (Fig. 1), it is necessary to preculture the parent strain for several generations in media containing low concentrations of asparagine, i.e., 50 μM or below, to achieve the results shown. The direct transfer of strain 25287 cells from cultures containing asparagine supplements above 100 μM results in poor or no growth of the subcultures provided with low concentrations of asparagine. Mutant strain 47 when cultured with similar conditions experiences only short lags before establishing a 60- to 70-min doubling time.

TABLE 2. *Asparaginase activity of strain 25287 and cytoplasmic asparaginase-deficient mutant 47*[a]

| Strain | Growth conditions | | Asparaginase activity (μmol per mg [dry weight] of cells per h) | |
|---|---|---|---|---|
| | Standard | Anaerobic, nutrient broth | 10 mM Asparagine[b] | 0.1 mM Asparagine[c] |
| 25287 | + | | 5.7 | 0.25 |
| | | + | 18.5 | 8.20 |
| 47 | + | | 0.0 | 0.07 |
| | | + | 18.7 | 19.30 |

[a] Suspensions of freshly harvested, washed cells were incubated at 37 C for 15 min in presence of 5% toluene.

[b] Asparaginase activity was determined by ammonia formation during a 15-min incubation of toluenized cells with 10 mM asparagine.

[c] Asparaginase activity was determined by the rate of aspartate formation over a 90-s period of incubation of toluenized cells with 0.1 mM [$^{14}$C]asparagine.

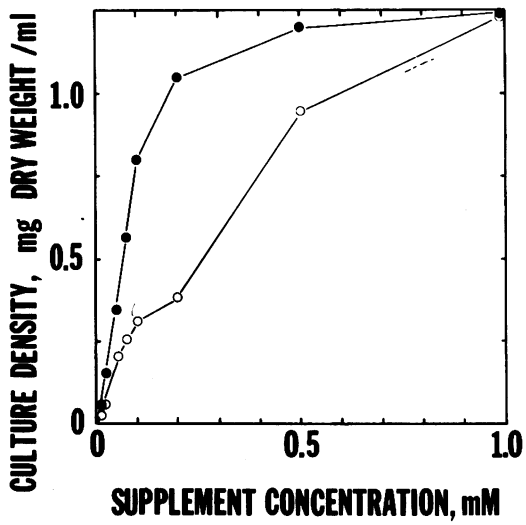


FIG. 3. *Efficiency of utilization of asparagine supplements by asparagine auxotrophs. Cultures* (○) *25287 and* (●) *47 were preconditioned and grown as described in Fig. 1. The points indicated refer to the cell mass achieved when growth ceased in cultures initially supplemented with asparagine concentrations shown.*

**Inhibition of cytoplasmic asparaginase activity.** *E. coli* can have two asparaginase activities (2): (i) a high-affinity ($K_m$ = 10 μM) periplasmic enzyme that is subject to catabolite repression (3, 4, 17, 20), asparaginase I, and (ii) a low-affinity cytoplasmic enzyme (3, 17, 20), asparaginase II. *E. coli* K-12 strains, in particular, have very little periplasmic enzyme when grown under conditions favoring catabolite repression (20). In our studies with strain 25287, asparagine deamidation by cells grown under the standard conditions is accomplished nearly exclusively by the cytoplasmic asparaginase activity (Table 2). The cytoplasmic asparaginase has an apparent $K_m$ of 3.5 mM for L-asparagine (Fig. 4).

The asparagine analogue, DONV, which has been shown to be a potent, competitive-irreversible inhibitor of the periplasmic enzyme, asparaginase II, (5, 7, 9) is also a competitive

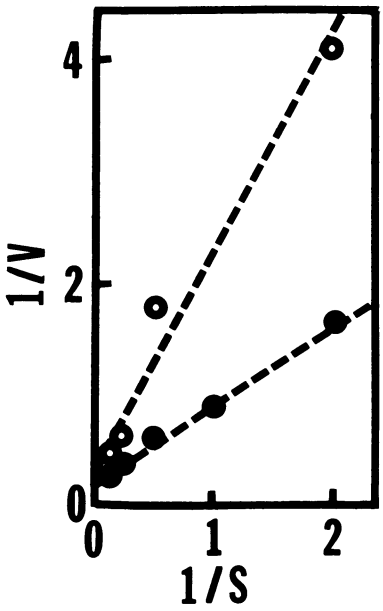


FIG. 4. *Double reciprocal plots of the concentration dependence of cytoplasmic asparaginase activity in the presence (●) and absence (○) of DONV. Strain 25287 cells harvested from a culture growing exponentially under standard conditions were washed and suspended in 0.01 M Tris-$KH_2PO_4$, pH 8.0, and 1 μM $MgCl_2$ to concentrations of 10 mg of protein/ml, approximately 20 mg of cells (dry weight) per ml. Toluene was added to a final concentration of 5%, and the suspension was incubated at 37 C for 30 min. The reaction mixtures contained asparagine at final concentrations indicated, 0.1 M Tris-$K_2HPO_4$, pH 8.2, and 1 μM $MgCl_2$. As indicated, DONV was present at a final concentration 5 mM. Assays were initiated by addition of warmed, toluene-treated cell suspensions to appropriately prepared assay buffers. Activity was determined by ammonia formation with 2 to 25 mM asparagine concentrations and aspartate formation with 0.5 mM to 2.0 mM asparagine concentrations as described in Materials and Methods. When necessary, the protein concentration of assay mixtures was adjusted such that no greater than 10% of the substrate was deamidated during the time course of the assay. V is velocity in micromoles per 30 min per milligram and S is asparagine concentration in millimoles per liter.*

inhibitor of the cytoplasmic enzyme. An apparent $K_i$ of 2.6 mM has been determined when the concentration dependence of asparaginase activity was assayed in the presence of 5 mM DONV (Fig. 4). Results of studies performed with a partially purified preparation of the cytoplasmic asparaginase (Fig. 5) indicate that DONV is, as defined by Baker (1), a competitive-irreversible inhibitor of cytoplasmic asparaginase activity. The analogue causes a progressive and irreversible inhibition of enzyme activity in the absence of asparagine and a nonprogressive and reversible inhibition in the presence of asparagine. We have not determined if the irreversible component of DONV inhibition is due to covalent reaction of the diazo group with the enzyme, as has been shown to be the case with the periplasmic asparaginase (9) and similarly as occurs between diazo-oxonorleucine and *E. coli* glutaminase (8). The 12.5% nonprogressive inhibition of cytoplasmic asparaginase activity by DONV observed in Fig. 5 is consistent with the kinetic parameters determined from Fig. 4.

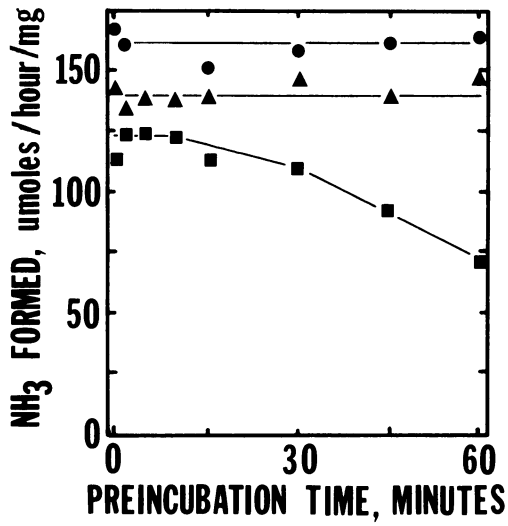


FIG. 5. *Inhibition of cytoplasmic asparaginase activity by DONV. The cytoplasmic asparaginase I was partially purified from strain 25287 to a specific activity of 165 μmol per h per mg by ammonium sulfate fractionation, diethylaminoethyl-cellulose and hydroxyapatite chromatography as described by Campbell et al. (2). The cells, harvested during logarithmic growth, were first osmotically shocked to remove periplasmic asparaginase II activity as described by Cedar and Schwartz (5). The enzyme used in the assay was preincubated in 0.1 M Tris-phosphate buffer, pH 8.2, at a protein concentration of 200 μg/ml at 37 C in both the presence and absence of 2 mM DONV. At the times indicated, asparaginase activities of the preincubated samples were determined by dilution of 0.1 ml into prewarmed assay buffer that consisted of 10 mM L-asparagine, 0.08 M Tris-phosphate buffer, pH 8.2, and 0.2 mM or no DONV. Asparaginase activity was measured by ammonia formation as described in Materials and Methods. Symbols: ●, no DONV during preincubation or assay; ▲, no DONV during preincubation, 0.2 mM DONV present during assay; ■, 2 mM DONV present during incubation, 0.2 mM DONV present during assay.*

**Effect of DONV on growth with asparagine as the nitrogen source.** The growth rate of cultures utilizing asparagine as the nitrogen source is immediately inhibited with addition of DONV (Fig. 6A). Although slightly progressive, the inhibition of the growth rate can be relieved at any time by a 1:10 dilution of the culture with fresh medium containing either asparagine or ammonium sulfate as the nitrogen source (Fig. 6B, C, D). A lag is observed in subsequent growth, the duration of which is dependent on the duration of exposure of the culture to the analogue. In contrast to the growth of subcultures utilizing ammonia as the nitrogen source, the growth of subcultures utilizing asparagine is again limited by the continued presence of the 10-fold diluted analogue. If the DONV-inhibited cells are removed from the medium and suspended in fresh media containing asparagine as the nitrogen source (Fig. 6E), complete growth of the culture results without appreciable lag. No apparent change was observed in cytoplasmic asparaginase activity of cells collected, toluenized, and assayed over the course of the experiments described in Fig. 6.

The effect of DONV on cell cultures does not appear to be irreversible as found with the partially purified cytoplasmic asparaginase (Fig. 5). This could possibly be due to the analogue not penetrating the cell while remaining an inhibitor of asparagine entry or to a protective effect of an asparagine pool inside the cell.

**Effect of DONV on growth of an asparagine auxotroph.** In contrast to the effects of DONV on cultures utilizing asparagine as a nitrogen source, growth rates of strain 25287 cultures dependent on asparagine supplements for requirement in protein synthesis are not inhibited with the addition of the analogue. Growth of the strain 25287 cultures in the presence of DONV is similar to growth of the asparaginase-deficient auxotroph 47. Greater terminal cell masses are observed for the strain 25287 cultures grown in the presence of the analogue than in parallel cultures not containing the analogue (Fig. 7). DONV in the absence

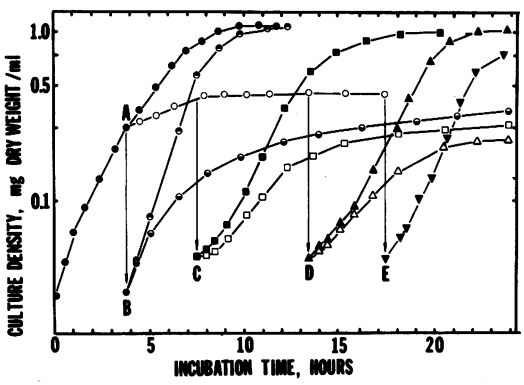


FIG. 6. *Inhibition of asparagine-nitrogen-dependent growth of E. coli by DONV. A culture of strain 25287 growing under standard conditions in minimal media containing 10 mM* L-*asparagine as the nitrogen source was divided (A) into two flasks that contained minimal medium and either (●) no further additions or (○) a final concentration of 2.5 mM DONV. At the times indicated by B, C, and D, cultures containing DONV were diluted 1:10 into fresh, warmed media containing (◒, □, △) 10 mM* L-*asparagine or 10 mM ammonium sulfate (◒, ■, ▲) as nitrogen source. At the time indicated by E, the DONV containing culture was washed and suspended in fresh, warmed media containing 10 mM* L-*asparagine as the nitrogen source.*

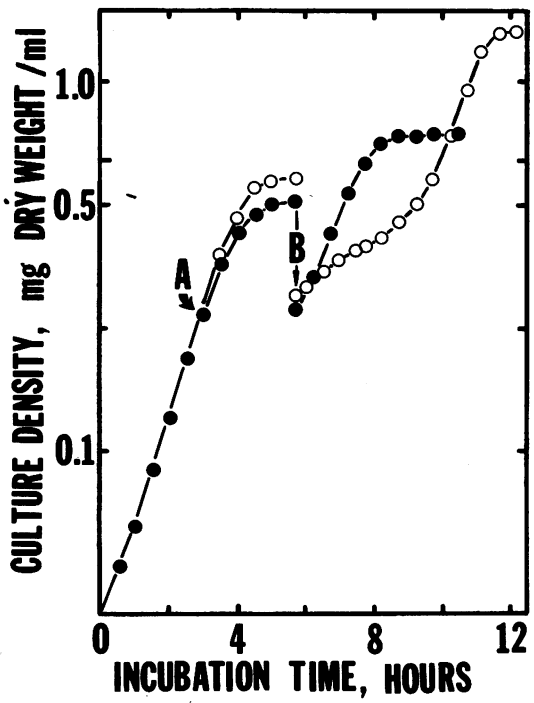


FIG. 7. *Growth of an asparagine auxotroph in the presence of DONV. A portion of a culture of strain 25287 growing under standard conditions in minimal media supplemented with 0.5 mM* L-*asparagine was transferred as indicated by A into a warm flask that contained a final concentration of 0.6 mM DONV. Growth was allowed to continue in both flasks until turbidities remained unchanged with time. The cultures were then diluted 1:2 with warm minimal media supplemented with 0.5 mM* L-*asparagine as indicated by B and culturing continued. The symbols represent culture containing before 1:2 dilution (●) no DONV, (○) 0.60 mM DONV.*

of asparagine does not relieve the auxotrophy of cells for asparagine. Cultures starved for asparagine in the absence of DONV immediately reestablish growth rates typical of the strain when diluted with fresh media containing asparagine. On the other hand, cultures starved for asparagine in the presence of DONV reestablish the typical growth rate upon dilution with fresh media after a lag period that is dependent on the concentration of the asparagine supplement present in the diluted media. In similar experiments, the asparagine-starved cultures were washed and suspended in fresh medium, and the lag period noted above remained but was less apparent; significant increases in terminal densities of subcultures from the DONV-treated cells were not observed.

**Metabolism of labeled asparagine by growing cultures of asparagine auxotrophs.** The utilization of uniformly labeled L-[$^{14}$C]asparagine supplements was followed with cultures of strain 25287 growing in the presence and absence of DONV and in the asparaginase-deficient strain 47 (Fig. 8). Strain 25287 cultured in the absence of DONV incorporates 5 to 10 times more label from asparagine carbons into trichloroacetic acid-precipitable materials during growth than parallel cultures grown in the presence of the analogue or cultures of the asparaginase-deficient strain 47. Furthermore, a significantly greater percentage of initial asparagine carbon can be recovered from the trichloroacetic acid-soluble material of either strain 47 cultures or strain 25287 cultures grown in the presence of DONV. These results are consistent with the interpretation that DONV and the mutation occurring in strain 25287 inhibit both the degradation of asparagine to aspartate and, consequently, the formation of the aspartate-glutamate family of amino acids and volatile respiratory products from this source.

The distribution of asparagine carbon into the amino acids of protein from cultures of strains 25287 and 47 grown in the presence of labeled asparagine supplements is shown in Fig. 9. Clearly, the mutation occurring in strain 47 prevents the distribution of asparagine carbon to amino acids for which aspartate serves as a precursor or metabolic intermediate. In similar preparations of amino acids from strain 25287 grown in the presence of DONV, the pattern of distribution of asparagine carbon was qualitatively the same as observed with strain 47. The fact that asparaginyl residues are deamidated during acid hydrolysis and are subsequently recognized as aspartate residues after chromatography does not interfere with the interpretation of the results. The presence of significant concentrations of labeled aspartate in cells during growth would be expected to be reflected in a significant labeling of the aspartate-glutamate family amino acids as observed with strain 25287 labeled in the absence of DONV.

## DISCUSSION

The original aim of this work was to investigate the utilization of exogenous asparagine by *E. coli* K-12 strains as an aid in establishing the significance and physiological role of systems mediating the entry of this amino acid. Roberts

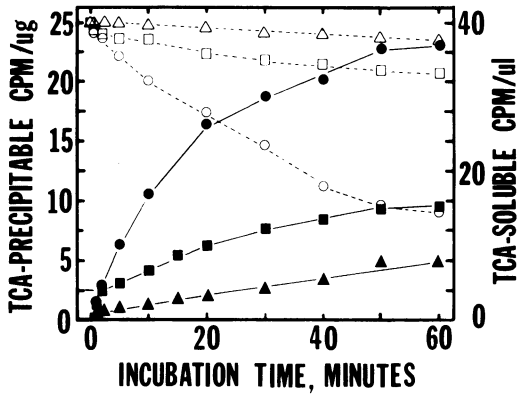


FIG. 8. *Utilization of labeled asparagine supplement by cultures of strain 25287 growing in the presence and absence of DONV and by the asparaginase-deficient strain 47. Portions of 3 ml were removed from exponential phase cultures of strains 25287 and 47 growing in the standard minimal media initially supplemented with 0.1 mM* L-*asparagine. The cells were washed and suspended in 10 ml of fresh minimal media containing either no DONV or 0.5 mM DONV and supplemented with 0.5 mM* L-*asparagine. The subcultures were grown for 30 min, and then 5 μliters of* L-[$^{14}$C]*asparagine (218 μCi/μmol, 50 nCi/μliter) were added and growth was continued under standard conditions. At the times indicated, 1-ml samples of the cultures were transferred to tubes containing 0.1 ml of 100% trichloroacetic acid. The solution was heated for 15 min at 50 C, vortexed, and filtered (18). Before the filters were washed, 0.1 ml of the filtered solutions was transferred to scintillation vials (trichloroacetic acid soluble). After being washed with 5 ml of 10% trichloroacetic acid, the filters were dried and transferred to scintillation vials (trichloroacetic acid precipitable). Radioactivity was determined in the presence of 5 ml of toluene-scintillator. Units refer to trichloroacetic acid-precipitable radioactivity per microgram of bacterial protein (solid symbols) and trichloroacetic acid-soluble radioactivity per microliter of culture fluids (open symbols); strain 25287 (circles), strain 25287 in presence of DONV (squares), and strain 47 (triangles).*

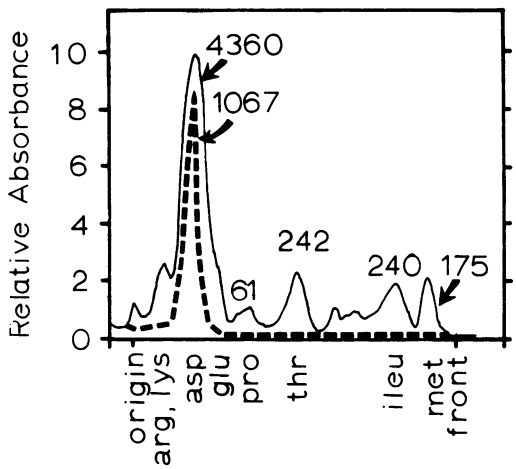


FIG. 9. *Densitometric comparison of autoradiograms prepared from one-dimensional chromatograms of acid hydrolysates of the chloroform extracted, acid-ethanol washed, hot trichloroacetic acid precipitates of cultures of strains 25287 (solid line) and 47 (broken line). Amino acid identification and radioactivity measurements, disintegration per minute, corresponding to numbers over peaks were made after the second dimension of chromatography and autoradiography. The cultures were grown under standard conditions for 60 min in the presence of 0.5 mM* L-*asparagine (0.05 mCi/mmol) after transfer of 3 ml of exponential phase culture growing in the presence of a 0.1 mM asparagine supplement into 7 ml of the labeling medium. The amount of hydrolysate spotted on each plate represented 100 μg of protein or 200 μg of cells (dry weight).*

et al. (18) have suggested from results of ammonia-sparing studies that asparagine, relative to aspartate or ammonia, is not a preferred source of nitrogen for *E. coli*. Our results indicate that the entry of this amino acid is dependent on the asparagine concentration of the medium below 5 mM and is growth rate limiting at all concentrations when used as the nitrogen source of cultures utilizing glucose as the carbon source. On the other hand, the entry of asparagine is not growth rate limiting when required to satisfy the auxotrophy of asparagine synthetase negative mutants provided the media contains an asparagine supplement of at least 25 μM. The observation by Cedar and Schwartz (4, 5) that asparagine-requiring auxotrophs require relatively high concentrations of asparagine for growth may be accounted for by either cytoplasmic asparaginase activity, repression of the high-affinity asparagine transport system, or a combination of both.

Asparagine supplements are not conserved by *E. coli* asparagine auxotrophs. It appears that asparagine molecules entering the cell in excess of the requirements for these amino acids in protein synthesis are rapidly deamidated to aspartate and ammonia rather than accumulated. Reduction of the cytoplasmic asparaginase activity of an auxotroph by either mutation or inhibition with the asparagine analogue, DONV, allows cultures to conserve their asparagine supplements. Cultures of the cytoplasmic asparaginase-deficient mutant, strain 47, in contrast to the asparagine-requiring parent strain, strain 25287, grow to saturation with asparagine supplements typical of the concentrations of amino acids used to support other auxotrophs. Our data indicate that approximately 131 μmol of asparagine is required for the synthesis of 0.5 g of cellular protein or 1 g of cells (dry weight).

The cytoplasmic asparaginase of *E. coli* strain 25287 has an apparent $K_m$ for asparagine of 3.5 mM. From a kinetic consideration this activity would not be expected to interfere with the endogenous asparagine pool that is approximately 0.3 mM in *E. coli* W 3110 cells harvested during exponential growth in a glucose-minimal medium (R. C. Willis and D. Keene, unpublished observation). The cytoplasmic asparaginase activity could, however, account for the high concentrations of asparagine required in media to repress the asparagine synthetase of *E. coli* K-12. For example, Cedar and Schwartz (14) found that the synthetase activity of cells cultured in 20 mM asparagine was only repressed 50% and that total repression required growth in the presence of 60 mM asparagine. Consistent with these observations, our results suggest that, until the cytoplasmic asparaginase approached saturation by asparagine, a significant increase in the size of the endogenous asparagine pool would not be expected and, consequently, effective repression of the asparagine synthetase would not be expected to occur. In contrast, culture of cells in the presence of 1 mM asparagine can repress the high-affinity asparagine transport system (Willis and Woolfolk, manuscript in preparation). This system appears to be responsible for the uptake of asparagine at low supplement concentrations, i.e., 25 to 100 μM. The result of a repression of the high-affinity asparagine transport system by culture of cells in 1 mM asparagine is poor, or there is no growth of cells after transfer to media supplemented with low asparagine concentrations (cf. Fig. 1), or, in the case of the asparaginase-deficient mutant, a lag period before the culture resumes the typical growth rate. Repression of the high-affinity transport system does

not result if cells are precultured or maintained in media supplemented with 50 or 100 $\mu$M asparagine.

The $\beta$-diazo analogue of asparagine, DONV, inhibits asparagine degradation at both the cellular and enzymatic level. This compound has been previously shown to provide a competitive and irreversible inhibition of the periplasmic asparaginase II (9). In our studies, DONV was found to be a competitive (apparent $K_i$ = 2 mM) and irreversible inhibitor of the cytoplasmic asparaginase I. Incubation of the cytoplasmic asparaginase I with DONV results in a time-dependent, irreversible inhibition of asparaginase activity. We have not determined if the irreversible effect is due to diazotization of the enzyme as found to be the case with the periplasmic asparaginase (9) or with 6-diazo-4-oxo-L-norleucine and the *E. coli* glutaminase (8). The cytoplasmic asparaginase is protected from the irreversible effects of the analogue by the simultaneous presence of asparagine.

In culture studies, DONV was found to be nearly as effective in promoting asparagine conservation as the genetic block found in a mutant strain deficient in the cytoplasmic asparaginase. Our data suggest that the inhibition of growth of asparagine-nitrogen-dependent cultures by DONV results from a time- and concentration-dependent accumulation of the analogue by the cells. Under conditions where growth of cultures is completely inhibited by the analogue, the asparaginase activity of the cells is not irreversibly inhibited. This result suggests the possibility that the enzymatic activity of asparaginase may be protected by a residual asparagine pool. Furthermore, the DONV pool of inhibited cells appears to be exchangeable with asparagine since dilution of inhibited cultures with fresh, asparagine-containing medium relieves temporarily the DONV-dependent inhibition of growth of the cultures. Total relief of cultures from the effect of DONV is only achieved by a complete removal of the analogue from the culture medium.

The suggestion that DONV affects the growth of the culture by entry into the cell and subsequent inhibition of the cytoplasmic asparaginase is consistent with our direct studies on the mechanism of asparagine transport carried out in conjunction with these metabolic studies and to be presented in detail elsewhere. That study has revealed two transport systems for asparagine, a very specific low-velocity high-affinity system responsible for the growth of asparagine auxotrophs at supplemental levels of asparagine (Fig. 4) and a less specific high-velocity system overlapping with aspartate, responsible for, but limiting the rate of growth of the culture on asparagine and related compounds as a nitrogen source (Fig. 2).

## ACKNOWLEDGMENTS

This research was supported by Public Health Service Predoctoral Research Fellowship award GM 42291 from the National Institute of General Medical Sciences awarded the senior author and by Public Health Service grant CA-08390 from the National Cancer Institute.

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